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CONTRACT NO: DAMD17-86-C-6042

TITLE: DRUG DEVELOPMENT AGAINST VIRAL DISEASES
(BIOLOGICAL TESTING)

(2)

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III. TESTING IN MURINE MODELS

A. THE CRIMEAN-CONGO HEMORRHAGIC FEVER VIRUS MODEL

1. Statement Of Problem Under Study

The purpose of this work was to conduct primary testing of antiviral compounds in a murine model of Crimean-Congo hemorrhagic fever virus infection.

2. Background And Review Of Appropriate Literature.

Infection of humans with Crimean-Congo hemorrhagic fever virus (CCHF) often results in a serious illness followed by death. It has been estimated that about 33% of hospitalized patients with a CCHF virus diagnosis die⁽¹⁾. The etiologic agent of CCHF is a single-stranded RNA virus of the *Nairovirus* genus in the Bunyaviridae family⁽²⁾. It is distributed throughout much of Africa, Europe and Asia. Virus strains from differing geographic regions have not been distinguished by those serologic techniques which have identified geographically distinct virus strains from other virus families⁽³⁾. The epidemiology of CCHF has been reviewed in detail by others^(4,5).

There is no widely used or widely accepted vaccine for CCHF. CCHF vaccine is not available commercially outside Bulgaria and the USSR. Administration of plasma from immune donors is considered by some to be the standard specific treatment for patients with CCHF⁽¹⁾. However, immunotherapy in CCHF is not without controversy. One view is that the variable results after human immunotherapy trials reflect the difficulty in getting donors with high antibody titers⁽⁹⁾. There are other problems. Immune plasma or globulin cannot be used prophylactically because the supply is limited and neutralizing antibody titers in convalescent plasmas are low. In actual practice, only patients with a well-established CCHF diagnosis receive immune plasma. In one study, only transient improvement in clinical status was noted without a clear effect on viremia⁽⁶⁾.

There has been limited experience with alternate forms of CCHF therapy. During a 1984 outbreak of CCHF at Tygerberg Hospital in South Africa, two different antiviral agents, ribavirin and human leukocyte interferon, were given. Ribavirin was used prophylactically in 6 of 9 CCHF case-contacts who had sustained direct blood exposure by various means including needle-pricks. Three individuals also received a short course of interferon. Of the 6 treated contacts, one (who did not receive interferon) developed a mild case of CCHF. The 3 untreated contacts developed severe CCHF⁽⁷⁾. South African patients who were treated with high doses of human leukocyte interferon either prophylactically or therapeutically (together with plasma) developed severe side-effects; the practice was stopped⁽⁷⁾. The results of this clinical trial did not provide an uncomplicated basis for continuing use of these two antivirals during future CCHF epidemics. There were no laboratory data from the South African study showing drug efficacy against CCHF virus infections.

Six years later, a brief report was given of the result of treating 12 CCHF patients with ribavirin in open-label trials in South Africa^(8, 9). When ribavirin therapy was started early (≤ 4 days post-onset), 0 of 7 patients died. However, of the seven survivors, only 2 had clinical laboratory markers associated with

... mortality in historical (i.e., untreated) controls. After late initiation of therapy (≥ 5 days post-onset), 3 of 5 patients (60%) died.

3. Rationale Used In Current Study.

This present research was to find a scientific rationale for use of antivirals in CCHF virus infections. The focus of the work was on ribavirin since it had been used in a human trial. Considerations involved in the design and execution of an antiviral drug study have been reviewed elsewhere (10).

According to prevailing criteria, CCHF virus infection is suitable for antiviral study. First, outbreaks of CCHF have been unpredictably sudden, frequent enough and of enough seriousness to warrant antiviral drug study. Secondly, the potential for antigenic or biologic variation among CCHF virus strains increases the need for a broadly active antiviral drug. Thirdly, this was a practical study because it could be incorporated into a broader study designed to find antivirals against a wide spectrum of virus diseases.

Proper models for CCHF infection have been difficult to establish because frequently used laboratory animals show little, if any, sign of infection or disease (11). Also, laboratory work with CCHF has been limited because accidental infections which have had serious or even fatal outcomes have too frequently happened (11). Also little is known about the human disease process. So, as has been done in similar situations, both the candidate compound and the experimental model itself had to be examined in this study (10). The experimental model used in these experiments was an infant mouse model based on intraperitoneal (ip) inoculation of CCHF virus.

4. Experimental Methods

a. Number and types of drugs tested. Approximately 500 drugs from 4 classes were tested. The four classes of drugs included acids and optically protected variants, amines and derivatives, nucleoside analogues with modified side chains and contaminant-free derivatives of natural products some of which were immunomodulators. Drugs were coded so laboratory testing was done with only solubility information. The chemical identity and the relationship between drugs were unknown by laboratory workers.

b. Model of CCHF virus infection. Drugs were tested for toxicity in 1-3 d old, Charles River strain CF1 random-bred mice at 50 mg/kg. Eight pups were inoculated ip with each drug. Those drugs which were not toxic were tested against CCHF, strain IbAr 10200, passage 11 or 12 in infant mouse brain (3). This virus strain was isolated from a pool of ticks in Ibadan, Nigeria and was chosen for biologic safety reasons because it had not been associated with either naturally occurring or laboratory-associated human disease.

Each drug was inoculated ip into infant mice in a volume of 0.075 ml. Forty-five minutes later, 50 LD₅₀'s of virus were inoculated ip in a volume of 0.075 ml. Placebo-treated mice were inoculated with tissue culture medium (DMEM). The virus was titrated simultaneously. In multiple drug dose tests, a total of 4 more daily drug doses was given after injection of virus.

...mice were housed and observed daily in a biosafety level 3+ animal facility modified especially for this work. Sentinel mice were tested serologically for intercurrent murine infections by the Yale University Division of Animal Care.

c. Biologic safety. Class 2 biological safety cabinets were used in a biological safety level 3 laboratory for manipulations involving infectious or potentially infectious materials. Individual workers were equipped with HEPA-filtered respirators and underwent serologic surveillance throughout the course of this project. Laboratory work was closely monitored by the University's Biological Safety Advisory Committee and its representatives and by the Safety Officer of U.S. Army Medical Research Institute of Infectious Diseases. Safety conditions on this project and in the University, at large, were reviewed in great depth by the Chairman of the Senate Subcommittee on Oversight Of Government Management of the Committee on Governmental Affairs, United States Senate (12).

d. Analysis of data. Geometric mean times to death were calculated for placebo (virus control) mice (VC) and drug-treated mice. The geometric mean time to death was equal to the n th root (where n =the total number of animals) of the product of each day with mortality raised to the power of the number of animals dying on that day. In this calculation, survival was defined as 28 d.

METHOD FOR MEASURING ANTIVIRAL DRUG EFFECT

$$VR = \frac{\text{GEOMETRIC MEAN SURVIVAL TIME (DRUG)}}{\text{GEOMETRIC MEAN SURVIVAL TIME (PLACEBO)}}$$

The virus rating of antiviral efficacy (VR) for each drug was equal to the ratio of the geometric mean time to death for each drug divided by VC (13). When a single positive drug was identified, that drug was incorporated into each test as a standard of test sensitivity. The data were analyzed as described in an earlier publication on CCHF virus from this laboratory (3).

e. Pathogenesis of CCHF in Infant Mice. Placebo-treated and ribavirin-treated mice were each divided into two groups, one of which was held for observation. Virus was titrated daily from the other group by inoculating SW-13 cells (supplied by Dr. D. Watts, U.S. Army Medical Research Institute of Infectious Diseases) with blood serum, liver, brain, spleen, and heart tissue homogenates. Tissues from eight animals were pooled and examined in four replicate cultures at each interval. Mice were first exsanguinated and then perfused with DMEM. Other tissues were removed, weighed, homogenized, clarified by light centrifugation and the supernatant fluid inoculated into freshly made cultures as described in earlier pathogenesis experiments (14). Virus titers were determined by fluorescence assay of infected cells under semi-solid overlay and expressed as FFU's/inoculum as described earlier (3). The variation between individual titers was less than 0.3 log₁₀.

Infected tissues were also examined by immunofluorescence. Sections of liver tissue from 6 mice were cut in 4-6 micron sections, mounted, fixed in acetone, stained with CCHF or control rabies virus antibody, washed in PBS, and stained with fluorescein-conjugated goat anti-mouse antibody (Antibodies Incorporated, Davis, CA). Co-localization of virus and specific cell types was by peroxidase staining of paraffin-embedded tissues using a commercial kit (ExtraAvidin Biotin Staining Kit, Sigma, St Louis, MO) and virus-specific or cell-specific monoclonal or polyclonal antibodies. Mac-1 antibody (monoclonal

clone M1/70HL to a surface antigen found on mouse macrophage cells) was purchased commercially (Boehringer Mannheim Biochemicals, Indianapolis, IN) (15). This antibody has been described as being weakly reactive with liver tissue (15).

f. Serology. Preparation and use of CCHF virus polyclonal antibody in IFA and neutralization tests was as earlier described (3). Antibodies for immunofluorescence observation included those described in an earlier publication and others from the reference collection of the Yale Arbovirus Research Unit (2). Neutralization tests for monitoring the purity and identity of ribavirin-resistant virus were done using constant antibody and varying virus dilutions. In this protocol, mice which escaped neutralization (i.e., *breakthrough* mice) were examined by IFA to establish the identity of the virus in the tissues.

Hybridomas producing monoclonal neutralizing anti-CCHF antibody were prepared after intrasplenic inoculation of virus using otherwise standard methods (16, 17). The monoclonal antibodies were isotyped using a commercial kit (Zymed Laboratories, Inc., San Francisco, CA). Screening was done by IFA and immunoprecipitation using a method modified from Mason (18). Pelleted (100,000 g for 2 h) radiolabeled (^{35}S) CCHF virus from CER cell culture supernatant fluids was solubilized in RIBB buffer (10mM Tris, pH7.5, 150 mM NaCl, 5mM EDTA, 1% Na deoxycholate, and 1% Triton X-100) containing protease inhibitors (PMSF 1mM, Aprotinin 10 $\mu\text{g/ml}$, leupeptin 2 $\mu\text{g/ml}$, benzamidine 2 $\mu\text{g/ml}$ and TLCK 10 $\mu\text{g/ml}$). Fifty μl of solubilized material were incubated overnight at 4°C with 1-2 μl of clarified monoclonal or polyclonal ascitic fluid. The immunoprecipitated material was sedimented with Protein A-Sepharose beads (Sigma) which had been washed 3 times previously with RIPA buffer (RIBB buffer with 0.1% SDS). The pelleted material was washed 5X with RIBB buffer and the proteins were released from the beads by incubation in sample buffer for 5 min at 100°C. The proteins were separated on a 10% acrylamide gel (19) which was processed for autoradiography using Kodak film.

5. Summary Of Results

a. Ribavirin in the CCHF infant mouse model.

When given in a single dose early after infection, ribavirin was an effective treatment in doses as low as 50 mg/kg in the CCHF model. In 19 tests using an exact 50 LD₅₀ virus dose, the mean GMTD was 15.2 \pm 0.5 days, an increase of 7.2 days ($p=0.0001$). Using a constant 50 mg/kg dose of ribavirin, a direct relationship (correlation coefficient ($r^2=0.88$)) existed between ribavirin GMTD and virus dose (25 to 200 LD₅₀'s). The lowest increase in GMTD was 3.2 days (200 \pm 80 LD₅₀'s; $p=0.002$) and the greatest, 12.0 (25 \pm 10 LD₅₀'s). (Table 1)

Using 50 LD₅₀'s of virus, the GMTD after multiple ribavirin doses (50 mg/kg, for 5 d) was higher (20.4 \pm 0.3) than after a single dose (15.2 \pm 0.5). The GMTD after multiple injections of 100 mg/kg was 21 \pm 0.4. However, a single 100 mg/kg dose was nearly as effective (20.2 \pm 0.4) as multiple doses (100 mg/kg). (Table 1) Beginning treatment on day 5, at the earliest time of onset of clinical signs, had no effect on either mortality or GMTD.

The GMTD was compared to the log protective effect (PI). The PI was a direct measure of reduced mortality attributable to ribavirin treatment. The correlation coefficient (r^2) was 0.76. A increase in GMTD of 3.2 days (range 3.1-3.5) was equivalent to a 1.7 PI.

These data were based on changes in GMTD. Experiments were then done to determine if increased GMTD, after ribavirin treatment was a result of decreased virus replication in a specific target organ.

Table 1. Ribavirin treatment of Crimean-Congo hemorrhagic fever (CCHF) virus-infected infant mice.

CCHF virus strain 10200 ^a		Ribavirin/Placebo treatment ^b		Outcome ^c	
Number of tests	Virus dose (LD ₅₀)	Drug dose mg/kg	Drug dose schedule	Percent mortality	Geometric mean time to death
6	50±15	Placebo	Single	100	8.0±0.6
19	50±15	50	Single	15	15.2±0.5
6	50±15	100	Single	2	20.2±0.4
2	25±10	Placebo	Single	100	8.6±0.4
2	25±10	50	Single	12	20.6±0.4
2	100±24	Placebo	Single	100	7.3±0.9
2	100±24	50	Single	37	14.4±0.5
2	125±33	Placebo	Single	100	7.6±1.2
2	125±33	50	Single	37	13.3±0.6
2	175±50	Placebo	Single	100	7.3±1.6
2	175±50	50	Single	50	11.0±0.8
2	200±80	Placebo	Single	100	7.0±2.2
2	200±80	50	Single	50	10.2±0.8
6	50±25	Placebo	Multiple	100	7.4±0.2
6	50±25	50	Multiple	12	20.4±0.3
6	50±25	100	Multiple	0	21.0±0.4

a Passage 6 in infant mouse brain tissue, ip inoculation

b Ribavirin (single) was given ip 45 min after virus or multiple with 4 additional doses on consecutive days. The placebo was culture medium (DMEM).

c 8-10 mice in each test group

b. Pathogenesis of CCHF virus in placebo-treated and ribavirin-treated infant mice.

The liver was the primary target organ in ip CCHF virus infection of infant mice. Virus titers were slightly higher in the liver than in the blood from day 3 to day 7 (death of the placebo-treated mice). Relative to virus growth in liver tissue, virus appeared very late after infection in other tissues including the brain and heart. Virus was not isolated from the spleen after day 2. The differences in virus titer between the liver and the brain, heart and spleen were significant ($p < 0.05$, paired t-test). However, the differences in titer between the liver and blood were not statistically significant.

Ribavirin-treated mice (single dose, 50 mg/kg) had significantly lower virus titers in liver tissue ($p = 0.007$, paired 2-tail t-test) and a significantly reduced viremia ($p = 0.007$, paired 2-tail t-test). Although virus growth in liver tissue was suppressed by ribavirin treatment, a large viremia was detected beginning on day 5. The titer of the viremia, in these experiments, seemed higher than the virus titer in the liver, but the

difference was not statistically different ($p=0.08$, paired 2-tail t-test). Despite the viremia, none of the treated-mice held for observation died. Mice were not examined after day 7.

The target organ was examined by both IFA and immunoperoxidase techniques using both frozen and paraffin-embedded tissue. Three d after inoculation of virus, CCHF virus antigen occurred in what appeared morphologically to be Kupffer cells lining the liver sinusoids of placebo-treated mice. Animals treated with a single dose of ribavirin had very little or no demonstrable CCHF antigen in these cells at this time. (Figures illustrating these data were presented in the Annual Report, 1989)

Later in placebo-treated animals, CCHF virus antigen was present in many clusters of hepatocytes widely distributed throughout liver tissue including occasional tissue macrophages. Infection of Kupffer cells was not prominent. CCHF antigen was only occasionally seen in liver tissue of ribavirin-treated animals, sometimes in a putative Kupffer cell or sometimes in a hepatocyte.

Kupffer cells were identified by immunoperoxidase staining on serial sections of paraffin embedded liver tissue sections using anti-Mac-1 antibody; infected cells were identified using anti-CCHF antibody. In consecutive tissue slices, some Mac-1 antibody-positive cells, i.e., Kupffer cells, were also positive with anti-CCHF antibody on day 3.

There was CCHF virus antigen in the liver tissue of animals treated with both single and multiple doses of ribavirin although the number of infected cells were few. Seven d after ip inoculation, infectious virus was isolated from the liver of mice treated with multiple doses of ribavirin (50 mg/kg for 5 days).

c. Effect of ribavirin on CCHF virus entry into the central nervous system.

In the experiments described above, evidence of CCHF virus infection of the liver was found in apparently healthy ribavirin-treated mice. Despite a relatively high viremia, infection did not spread to the brain or other organs. This result was different from that in placebo-treated animals where viremia was followed by infection of other organs (brain and heart).

These results suggested that the protective effect of a single dose of ribavirin (50 mg/kg) diminished with time. Therefore, the experiment was repeated, focusing only on viremia in ribavirin-treated mice and using a higher ribavirin dose (100 mg/kg). Additional doses of ribavirin were given on days 5 and 9. Virus isolations were not done before day 6. Viremia (\log_{10} FFU/ml) was found on days 6 (3.6 ± 0.2), 7 (4.5 ± 0.2), 8 (2.6 ± 0.2), 9 (2.4 ± 0.3) and continued at that level through day 11. On day 12, the titer (\log_{10} FFU/ml) decreased to 1.3 ± 0.3 ; virus was not found on days 13 or 14. Despite the relatively prolonged viremia, virus was isolated only from the liver (days 6-13), not from the brain, heart or spleen. Titers of liver virus were not determined. Mice held for observation did not die.

Liver tissue from CCHF virus infected, ribavirin-treated mice (single dose, 100 mg/kg) was titrated daily by ip subinoculation into infant mice. The peak titer in the liver was found on day 8. Eight ribavirin-treated mice (single dose, 100 mg/kg) without clinical signs of illness were necropsied on the eighth day after ip virus injection (50 LD₅₀). Virus was found in pooled serum and supernatant fluid from tissue homogenates of pooled liver but not other organs including brain, heart, and spleen. The infectious virus titer (\log_{10} LD₅₀/ml) in the liver was 4.0 and 2.3 in the serum as determined by ip inoculation of infant mice. Virus from liver tissue of ribavirin-treated mice grew very poorly in CER or SW-13 cells. For

individual wells of Lab-Tek slide trays containing 5×10^4 CER cells, 2-10 positive cells were observed by IFA. Results were similar with SW-13 cells. Residual ribavirin in the tissue homogenates could have inhibited virus growth *in vitro*. Therefore, the virus was pelleted from the supernatant fluid by ultracentrifugation (100,000g, 60 min), but virus infection of cultured CER cells was not increased. Because of the variability between individual cultures in the number of infected cells, it was impossible to determine *in vitro* ribavirin sensitivity reliably.

Attempts were made to determine ribavirin sensitivity *in vivo*. The original liver tissue virus from ribavirin-treated mice was used as source in an *in vivo* ribavirin drug test. Mice were infected with liver virus (50 ip LD₅₀) and treated with a single dose of ribavirin at either 50 or 100 mg/kg. The GMTD in mice treated with 50 mg/kg was unchanged from the placebo controls (mortality 8/8) and the GMTD in mice treated with 100 mg/kg was increased by 1.6 days (mortality 8/8). Eight of eight (8/8) placebo-treated animals died with an GMTD of 8.1 days.

Virus isolations were done by ip inoculation of infant mice using pooled tissues from two mice taken on days 7 and 8 from the two groups, one treated with 100 mg/kg and one placebo-treated. Virus was isolated from the liver and serum in both treated and placebo-treated mice on days 7 and 8 but not from other organs on either day. In treated mice (100 mg/kg), virus titers (log₁₀ LD₅₀/ml) were as follows: Day 7, liver, 4.2; serum 2.9; Day 8, liver 4.5, serum 2.7. Virus titers in placebo mice were as follows: Day 7, liver 5.7; serum 5.3; Day 8 liver 5.2; serum 5.3. These data show that ribavirin treatment (100 mg/kg) reduced virus titers in treated animals. In contrast to an earlier experiment with the parent virus, treatment with ribavirin at 50 mg/kg had no effect on virus titers (data not shown). Despite the relatively high viremia in placebo-treated mice (5.3 log₁₀ LD₅₀/ml), virus was not isolated from other tissues (brain, heart, spleen). This suggested a marked hepatotropism for the injected virus.

Three blind passages of the hepatotropic virus through placebo-treated mice resulted in a loss of strict hepatotropism. After three ip passages of liver tissue virus harvested on day 7, sick infected mice were necropsied. Isolated virus had the following titers (log₁₀ LD₅₀/ml): liver (6.1±0.2); serum (5.6±0.2); brain (7.1±0.2); heart (6.6±0.2). Strict hepatic tropism was no longer in evidence.

d. Ribavirin analogues in the CCHF model. Three types of ribavirin analogues were tested: (1) those with changes in the heterocyclic moiety; (2) those with changes in the glycosidic moiety; (3) C-nucleoside analogues. Three analogues closely related to the parent ribavirin (VR, 2.47±0.70) had similar efficacy: (1) ribavirin triacetate (VR, 2.30±0.07) (2) ribamidine (2.63±0.45) and (3) AVS 4071, 1-beta-D-ribofuranosyl-1,2,3-triazole-3-methylamidate (VR, 2.3). Tiazofurin triacetate (VR, 1.2) and seleanzofurin (VR, 1.5±0.14) were less active in the CCHF model. Tiazofurin (VR 1.0) and the seleanzofurin 5' monophosphate (VR, 0.9) were inactive. Additional modification of tiazofurin triacetate, seleanzofurin, and seleanzofurin 5' monophosphate resulted in either unchanged efficacy or toxicity problems.

6. Discussion And Conclusions

This research was to test ribavirin and other compounds for antiviral activity against CCHF virus to support intervention with ribavirin during human CCHF virus outbreaks. We used an infant mouse model because the virus does not obviously infect other commonly used laboratory animals. As a result, some classes of drugs (i.e., some immunomodulators) could not be reliably tested in our model. In addition, there is concern that metabolism of ribavirin may be different in infant mice and in primates.

On the other hand, finding the early association of CCHF virus with Kupffer cells supplied significant evidence suggesting the model bears relevance to human disease. A series of published autopsy findings was summarized by Hoogstraal in 1979 [4]. After extensive pathologic examination of many cases of fatal CCHF infection over a four year period, Karmysheva concluded that CCHF virus multiplied in cells (especially Kupffer cells) of the reticulo-endothelial system, in which specific antigens were detected by immunofluorescence.

Published proof of CCHF virus growth in Kupffer cells is in agreement with our present research findings. That is reassuring since some murine coronaviruses and some arboviruses have a liver tropism in infant mice similar to that shown in our experiments 20, 21, 22. Because of that, we have gone to elaborate lengths to eliminate intercurrent infection or contamination of reagents as an explanation for the experimental results. Using a CCHF virus neutralizing monoclonal antibody to confirm identity of resistant virus was one step in establishing its purity and identity.

Our data reported in detail in previous Annual Reports suggest that ribavirin-resistant virus was propagated in the liver of CCHF virus infected, ribavirin-treated mice. A dose regimen (single dose, 50 mg/kg) that, with the parent virus, reduced virus titers and mortality was ineffective using virus harvested from the liver of ribavirin-treated animals. Nevertheless, an increased dose of drug (single dose, 100 mg/kg) did, indeed, substantially reduce virus titers in both the liver and serum even though mortality was not reduced. Repeated passage of the liver virus in ribavirin-treated mice (multiple dose, 100 mg/kg) resulted in a loss of infectivity. These latter two observations suggest that, while partial resistance was apparent, it may not be real. What is seen as partial resistance in this model may be related to a pharmacologic property of ribavirin unique to infant mouse tissue. For example, infant mouse liver cells could become resistant to the effects of low doses of the drug thereby making sensitive virus appear resistant as postulated elsewhere.

Evaluation of ribavirin analogues and other antiviral compounds in the CCHF model became more important because alternative drugs or therapeutic procedures might be needed in clinical settings to minimize development of resistance. Our results can be put into the framework of ribavirin structure-activity relationships which have been earlier evaluated [29]. We are in agreement with previous experiments with other viruses in which addition of an amide group ($C=O$ to $C=N$) to reduce toxicity did not change ribavirin efficacy. However, addition of an amidine group ($N-CH_2-CH_2-CH_2$) to reduce toxicity destroyed drug efficacy. Adding an $-OH$ to the amidine group to reduce toxicity and increase solubility did not restore efficacy. One possible explanation for loss of activity is that the amidine group inhibits ribavirin phosphorylation.

Our results with ribavirin C-nucleoside analogues are different from those with other arboviruses [reviewed in 23]. We did not find that ribavirin triacetate was significantly more effective than ribavirin. In the CCHF model, tiazofurin analogues (AVS 111, AVS 257) are inactive whereas selenazofurin analogues (AVS 253, AVS, 3705) are active. We cannot address synergism in the CCHF model because combination therapy was not used.

The other group of nucleoside analogues active in the CCHF model were N-oxides of adenosine. It was not altogether surprising that both ribavirin and adenosine analogues were active in the CCHF model. Molecular models have confirmed the structural similarity between these two compounds although the resemblance of ribavirin to adenosine is not readily apparent.

B. THE LCM VIRUS MODEL

1. Statement Of The Problem And Rationale Used.

Antivirals for arenavirus infections remain a high priority. The prototype for this group of infections may be Lassa fever. Lassa fever is an acute infection characterized by fever, headache, myalgia, pharyngitis, vomiting, a relatively mild hemorrhagic diathesis, and a capillary leak syndrome with facial edema, adult respiratory distress syndrome, shock and multiple organ failure. The disease in experimentally-infected rhesus monkeys closely resembles that in humans. Ribavirin treatment initiated as late as 5 days after virus prevented lethal infection, reduced cell injury as reflected by serum transaminase levels, and abrogated viruria, inspiring open-label trials, in which mortality in treated patients was compared with that in historical controls. Despite shortcomings in design and analysis, a convincing case was made for efficacy of ribavirin. Antiviral treatment of experimental and human Lassa fever has not been associated with late encephalitis or encephalopathy, as described in the other arenaviral hemorrhagic fevers (reviewed in [9]).

A murine model of arenavirus infection was used to screen drugs for antiviral efficacy. 481 drugs were tested in the LCMV model. The methods and calculations for determination of efficacy were done by procedures described in detail above for CCHF virus.

2. Description Of The LCMV Model

Adult mice are inoculated with 50 mg/kg of drug i.p. in a volume of 0.4 mls. Forty-five minutes later, mice are inoculated with 50 LD50's of LCM virus (LCMV) i.p. The virus strain was propagated by intracerebral passage in inbred C3H mice. Random bred CF-1 mice from Charles River were used for drug tests. The identity of the virus strain was monitored by examination of infected mouse tissue by immunofluorescence.

3. Histopathology Of LCMV Infection

Adult random-bred mice inoculated with 50 LD50's of LCMV were examined on days 10 and 11 post inoculation. The details of our studies were presented in earlier Annual Reports. In summary, mice were suffering from severe, multisystemic disease, with necrotizing inflammation of lymphoid tissues, parotid salivary glands, pancreas, splenic red pulp, liver, intestine and mesentery. They also had mild focal

neutrophils. The majority of leukocytes, regardless of type, in all tissues examined were undergoing necrosis. No lesions were found in submaxillary or sublingual salivary gland, kidney, heart, eye, lacrimal gland, thyroid, trachea, or lung. Lesions were consistent with visceral LCM disease as described elsewhere (24).

4. Distribution Of LCMV Results

The mean VR score and the mode of distribution of all drugs tested 1.0 and 1.077 respectively. The relationship between VR score and percentile ranking was presented in earlier Annual Reports. VR scores of 1.5 or higher were in the 95% of all drugs tested.

5. Analysis Of Reproducibility And Variation.

Variation in the test system was low. The standard deviation of ribavirin (AVS#1) was 0.4. In the CCHF system, the standard deviation of the VR+ AVS #1 was 0.6. Thus, there was less variation in the LCM model, but there was also less consistent positive effect on survival.

6. Drugs Effective In Both The LCMV And CCHF Models

Thirty-one drugs were identified as being of potential interest for additional tests of efficacy. Ribavirin was effective in this model although the triacetate analogue (AVS 206) was more effective than the parent or any other ribavirin analogue tested. The VR scores ranges from 1.2 to 1.6 depending upon virus dose used and the number of drug doses given.

Aside from ribavirin, the most promising, in terms of VR scores, was AVS# 4070 which had mean VR's of 1.7 and 1.8 against test doses of 100 and 63 LD50's respectively. This drug had a higher VR score in the LCMV model than we observed with any other drug including ribavirin (AVS# 1) or any of its tested analogues.

There were 6 drugs which were effective in both the LCMV and the CCHF models. In addition to ribavirin, (AVS#1, 206), there are 4 other drugs. They included AVS #4071, 4720, 4796 and 4217. Data on these individual drugs were presented in detail in previous Annual Reports (1989 & 1990).

C. THE VACCINIA VIRUS MODEL

1. Statement Of The Problem And Rationale Used.

There is no evidence that the smallpox eradication program has been less than a total success. Nevertheless, the military continues to immunize its personnel with vaccinia virus. Because of the dearth of naturally occurring cases, the risk of vaccine-induced disease has begun to surpass the risk of naturally occurring disease. This is particularly true since a large closed-population of military personnel is being actively immunized. Vaccine-induced side effects become both a significant disease burden and financial liability. A high priority is development of an antiviral drug which could mitigate against the side effects frequently seen after vaccination. Toward this end, we have tested selected antiviral compounds and immunomodulators for their antiviral efficacy against vaccinia virus.

There are other public health reasons for developing antivirals effective against vaccinia virus induced disease. The use of live recombinant vaccinia virus strains which express immunoreactive epitopes from pathogenic agents, holds great promise as a means of immunoprophylaxis against a variety of human diseases²⁵. However, the risk of disease from the vaccinia vector itself remains substantially untested and therefore potentially significant²⁶. Development of antivirals could provide an additional safety margin for that time when such recombinant vaccinia vaccines are in wide use both in military and civilian populations. The testing system itself becomes a valuable adjunct in safety tests of vaccine constructors. We have studied such a testing system.

2. Experimental Methods

The history and evolution of the tail lesion model used in our studies is as follows. Boyle²⁷ first described tail lesions in mice infected with vaccinia virus and the effect of some antivirals in reducing the number of tail lesions. DeClercq²⁸ compared polyanions with interferon to determine their effect on vaccinia virus lesion formation on the tails of mice. Smejkal et al²⁹ determined antiviral and interferon-inducing activities of three benzo(c)fluorenone derivatives in mice. Moreover, they introduced the staining of tail lesions with 1% fluorescein and 0.5% methylene blue. Jacoby et al.³⁰ studied the pathogenesis of lesions produced by vaccinia virus by virological, morphological and serological methods. They found the primary lesions were typical pocks characterized by sequential development of epidermal necrosis, vesicle formation and ulceration, and by dermal inflammation dominated by mononuclear cells. Seroconversion was shown on day 8 after infection.

Our studies have focused on variables which have not been analyzed in previous studies of the tail lesion model including the effect of virus strain, mouse strain, age and weight on induction of tail lesions. In addition, we have tested several different categories of antivirals for efficacy in this model.

3. Results

a. *Vaccinia virus strains and tail lesions.*

The number of viral PFUs required to induce a tail lesion was determined for each virus strain after inoculation of the highest possible virus dose (Table 2a) or after a standard amount of virus at the lowest possible comparative dose (Table 2b). The four virus strains tested in CD1 mice were of markedly different virulence as measured by induction of tail lesions regardless of virus dose. The wild type and the Lister strains were clearly the most virulent requiring fewer PFUs to produce a lesion than the other virus strains regardless of the magnitude of the inoculum. The 3PP strain was the only one in which the magnitude of the inoculum had an apparent effect on the number of tail lesions observed. After a low dose inoculum, fewer PFUs (284, $10^{2.5}$) were required to produce one tail lesion whereas after a high dose inoculum, 62,797 ($10^{4.8}$) PFUs were required to produce one lesion. We have no explanation for this anomaly. In contrast, regardless of virus dose inoculated, the plasmid strain was of reduced virulence perhaps because it lacked a functional TK gene. 17,942 ($10^{4.3}$) PFUs were required to produce a single lesion with this construct. Lower virus doses did not produce lesions. The Copenhagen strain (alternately designated "Koppe") was of intermediate virulence requiring between 733 ($10^{2.9}$) and 995 ($10^{3.0}$) PFUs to produce a lesion. Since this strain was of intermediate virulence, it was used in subsequent experiments.

A. DAY 7 LESIONS AFTER INFECTION WITH HIGH DOSE VIRUS.

VIRUS STRAIN	VIRUS TITER (PFU'S/0.1)	PFU'S INOCULATED	LESIONS (MEAN)	PFU/LESION
WILD TYPE	398	398	46±11	9
LISTER	3,981	3,981	66±16	60
COPENHAGEN	63,095	63,095	86±14	733
PLASMID	25,119	25,119	1.4±1.4	17,942
3PP	25,119	25,119	0.4±.4	62,797

B. DAY 7 LESIONS AFTER INFECTION WITH LOW DOSE VIRUS.

VIRUS STRAIN	VIRUS TITER (PFU'S/0.1)	PFU'S INOCULATED	LESIONS (MEAN)	PFU/LESION
WILD TYPE	398	398	46±11	9
LISTER	3,981	398	6±1.6	66
3PP	25,119	398	1.4±1.4	284
COPENHAGEN	63,095	398	0.4±.4	995
PLASMID	25,119	398	0.0	∞

b. Relation between vaccinia (Koppe) virus dose, neurovirulence, and tail lesions.

There were two questions for which we sought initial answers. First, was there a relationship between PFU titer and mouse intracerebral neurovirulence and secondly, was there a linear relationship between virus PFU dose and the mean number of tail lesions produced?

For the Koppe virus, a PFU titer of $10^{3.9}$ corresponded to a suckling mouse intracerebral LD₅₀ titer of $10^{3.7}$ thereby establishing for this virus strain a close relationship between infant mouse neurovirulence and PFU titer. Dilutions of stock virus ranging from undiluted stock to 1:100 were tested for lesion production after tail vein inoculation of young adult CD1 mice (19-20 grams). The mean number of lesions actually counted ranged from 70 ± 15 (undiluted virus stock) to 5 ± 10 (1:100 virus dilution). R-squared, after linear regression of virus dilution on the number of lesions, was 0.942. Thus, over this virus dose range, there was a linear relationship between PFUs and lesions counted.

c. Effect of mouse age and strain on induction of tail lesions.

The effect of mouse age on tail lesions was determined by repeating the experiment described above using groups of ten CD1 mice of 19.5 grams and 34.6 grams mean weight inoculated with $10^{3.2}$ PFUs (1:5 dilution of the stock virus). The mean number of lesions counted was 51.2 ± 13.8 ($\sqrt{x^2} = 6.911 \pm 9$) for the 19.5 gram mice and was 28.6 ± 3.9 ($\sqrt{x^2} = 5.3 \pm 4$) for 34.6 gram mice. At a lower dose of virus (1:100 dilution of the stock virus), the mean number of lesions was 2.8 ± 1.1 ($\sqrt{x^2} = 1.6 \pm 3$) and 4.8 ± 0.75 ($\sqrt{x^2} =$

19.5 and 34.6 gram mice respectively. Although there was no strong evidence of an age effect, all subsequent experiments were done in young adult mice between 19-20 grams.

Four different mouse strains were tested for development of tail lesions using the range of virus concentrations described above. The dose responses were calculated for each mouse strain and compared by paired t-test for significance of differences. No significant differences were observed among the mouse strains tested at any of the time points (days 6, 7, 10) used for counting of tail lesions. CD1 mice were used in subsequent experiments.

d. Vaccinia virus antigen (Koppe and plasmid strains) and tail lesions.

Vaccinia virus antigen (Koppe strain) was localized by staining of transverse annular sections of tail tissue by indirect immunofluorescence. In the earlier stages after inoculation (up to day 3), antigen was found only in isolated foci of cells surrounding individual hair follicles. These locations were extremely focal and required examination of nearly the entire tail. Between days 3-5, antigen was found less frequently in this band of cells but appeared more diffusely distributed in the dermis. On days 6-8, antigen could be detected in focal areas of the epidermis in massive concentrations. At this time and in the area of antigen concentration, infiltrating inflammatory cells were observed. A similar early distribution of antigen was detected after inoculation of the plasmid strain. However, antigen spread beyond the dermis was never detected. Thus, development of tail lesions was associated with spread of virus antigen from an early focus of infection in the dermis to a more pronounced, but still focal, infection of the epidermis. Virus antigen was not prominently associated by immunofluorescence with sebaceous glands in the tail, muscle tissue, or other organs examined including kidney, lung, liver and brain.

e. Active chemotherapeutic agents in the vaccinia tail lesion model.

Most drugs tested in this model gave VR scores of 1.0. Drugs with VR scores of 1.4 were in the highest 90% percentile of all drugs tested. The potency of the chemotherapeutic antivirals which were active in inhibiting formation of mouse tail lesions following vaccinia virus infection (Koppe strain) is summarized in Table 3. Active compounds include ribavirin, ara A, adenosine N'-oxide and several of its analogues. The relationship between *in vitro* activity and *in vivo* activity was excellent for adenosine N'-oxide, but such correspondence was not absolute for other compounds. For example, ara A in cell culture was not particularly potent with a VR of 0.8 to 1.4. (A VR of 1.0 means no drug effect.) Yet, in the mouse model, depending upon the virus dose and the virus dose, the VR score ranged from 0.9 to 3.7 suggesting that the drug was more useful in animals than the tissue culture data suggested. Despite this promise, the therapeutic index of ara A is low (6-40) and alternative chemotherapeutic agents might be more useful. The therapeutic index is a ratio of efficacy to toxicity and is a measure of safety. Compounds were identified with a high therapeutic index, a high VR score in both cell culture and the tail lesion model. Included, in particular, is analogue of adenosine-N'-oxide such as AVS 4224, a pro-drug with a substituent 3,4-dimethyl group.

Table 3. Summary of Active Chemotherapeutic Agents in the Vaccinia Virus Tail Lesion Model

AVS #	Therapeutic Index (tissue culture)	VR (range in tissue culture)	VR § (range in tail lesion model)	Name or type of compound
1985	72-5650	3.4->7.2	1.0-2.3	adenosine-N'-oxide
Pro-drugs of adenosine-N'-oxide:				
1986	>487	3.4->7.2	1.0-1.3	substituent: 3-methyl
2911	>181, >310	>1.9->2.1	1.1-1.6	substituent: 2-nitro
3607	992->1000	>2.4->2.7	1.2-1.5	substituent: alpha-methyl
3679	11167-10000	2.8-3.3	0.6-2.0	substituent: 4-methoxy
4224	>996, >1762	>2.6-2.8	2.5-3.6	substituent: 3,4-dimethyl
Compounds other than adenosine-N'-oxide analogues:				
1752	6-40	0.8-1.4	0.9-3.70	adenosine arabinoside (ara A)
001	ND†	ND	0.7-2.0	ribavirin
7118	ND	ND	1.4	rifampicin
1214	50, 119	ND	4.3	5'-deoxyadenosine dialdehyde

†ND means not done.

§The usual drug schedule was one dose prior to virus inoculation followed by 5 subsequent daily doses. The mean number of tail lesions was equal to the average of $\sqrt{X^2}$ where X^2 is the number of lesions per mouse. The virus rating (VR) was a ratio of the average number of lesions in mice given DMEM as control divided by the average number of lesions in the drug-treated animals.

f. Immunomodulators in the vaccinia virus tail lesion model.

An alternative antiviral therapy involves the use of immunomodulators to inhibit virus spread. Combined therapy could be useful in mitigating against selection of drug-resistant virus strains. Five immunomodulators which have been widely studied elsewhere^{31, 32, 33, 34}, were also studied in the present experiments. Of the five, four were found to be efficacious in significantly inhibiting tail lesions in the vaccinia virus mouse model. These include quinolinamine (AVS 1018), amplitgen (AVS 2149), recombinant IL2 (AVS 5079), and poly ICLC stabilized for injection (AVS 1761). At the doses used, exogenous recombinant IL2 had no effect on tail lesion formation. Tails from selected groups of mice treated with quinolinamine and amplitgen were examined by indirect immunofluorescence for detection of vaccinia virus antigen. Although antigen was found in the dermis in the early days after infection, virus spread to the epidermis was not found in contrast to control mice. These results are in accord with the hypothesis that lesion formation is associated with spread of infection from the dermis to the epidermis. In addition, treatment with these two immunomodulators appeared to have specifically inhibited virus growth in epidermal tissue.

Table 4. Immunomodulators in the Vaccinia Virus Tail Lesion Model

Drug	DRUG DOSE†	VIRUS DOSE log ₁₀ PFU'S (KOPPE)	CONTROL MEAN LESIONS $\sqrt{X^2}$ ‡	DRUG MEAN LESIONS $\sqrt{X^2}$	VRV CONTROL + DRUG	P (t-TEST PAIRED ONE-TAIL)
quinolinamine	300 mg/kg	3.7	7.375± 1.034	0.333± .167	22.1	0.0001
quinolinamine	10 mg/kg	3.8	7.604± .708	3.403± .588	2.34	0.0003
IL 2, (recombinant)	2.5 x 10 ⁴ units	3.8	7.604± .708	6.473± .724	1.17	0.526
recombinant interferon	10 ⁵ units	3.8	7.604± .708	2.54± .915	2.99	0.0054
ampligen,	100 µgms	3.8	7.604± .708	1.631± .62	4.66	0.0003
ampligen,	50 µgms	4.3	10.44	3.06	3.41	0.0002
ampligen,	25 µgms	4.3	10.44	3.31	3.18	0.0001
poly ICLC, stabilized	20 µgms	3.8	7.604± .708	1.603± .503	4.74	0.0001

†The usual drug schedule was one dose prior to virus inoculation followed by 5 subsequent daily doses

‡The mean number of tail lesions was equal to the average of $\sqrt{X^2}$ where X^2 is the number of lesions per mouse.

VRV The virus rating (VR) was a ratio of the average number of lesions in mice given DMEM as control divided by the average number of lesions in the drug-treated animals.

4. Discussion And Conclusions

Vaccinia virus strains and constructs differ greatly in the number of PFUs required to produce tail lesions in the vaccinia virus mouse model. The pathogenesis of lesion formation appeared to involve virus spread from an initial focus in specific cells surrounding hair follicles to other concentrated areas of the dermis and finally, at the time of lesion development, to the epidermis. Antivirals which suppressed tail lesions, to a greater or lesser degree, included ara-A, ribavirin, rifampicin, adenosine N'-oxide and selected analogues. Immunomodulators, including ampligen and recombinant interferon suppressed lesions at very low doses. Spread of virus infection from the dermis to the epidermis was inhibited as determined by

...ence. These studies in the tail lesion model have suggested drugs which could be tested in primate models of vaccinia virus infection. In addition, these studies provide additional data on a model which may be a useful adjunct in safety testing of recombinant vaccinia virus vaccines.

III. TESTING IN PRIMATE MODELS

A. YELLOW FEVER AND ANTIVIRAL DRUGS

1. Background and statement of the problem

Preventive control of human disease by an antiviral substance can be best considered when it has a relatively broad spectrum of activity. Otherwise, the time delay involved in identification of the causative agent may render application of an antiviral impractical for prevention. Ribavirin is such an antiviral drug. In tissue culture and in rodents, it has broad antiviral activity. However, efficacy against flavivirus infection has not been convincingly demonstrated in laboratory infected primates. One goal of the work in this section was to test ribavirin for antiviral activity against yellow fever virus infection.

2. Summary of yellow fever-ribavirin tests.

a. Drug treatment at the time of and after virus exposure.

Squirrel monkeys, *Saimiri sciureus*, were inoculated subcutaneously with 1000 LD50 of yellow fever virus, Dakar 1270 strain, in the 8th mouse brain tissue passage. Ribavirin (50 mg/kg) was given subcutaneously at the time of virus inoculation and daily thereafter. Drug treatment had no effect on mortality. One of two virus infected animals survived whereas 1 of 3 drug treated animals survived. The geometric mean time to death for the control animals was 9.2 days compared to 9.4 days for drug-treated animals. The mean peak viremia level (day 3 after virus inoculation) in control animals was 6.8 log PFU in contrast to 5.1 in treated animals. Surviving control animals had slightly higher HAI antibodies but neutralizing antibodies were comparable. Alkaline phosphatase levels were elevated in the control animals, but only on day 3. This change was not correlated with death. One control animal treated with drug alone did not seroconvert.

b. Drug treatment before and after virus exposure

In a second experiment with squirrel monkeys, drug treatment (50 mg/kg) was initiated 3 days prior to virus exposure and continued for 8 days after virus infection. Prolonged survival, but no statistically significant reduction in mortality was seen in ribavirin-treated animals. Five of six (83%) animals infected with yellow fever virus alone died whereas 2/5 (40%) animals treated with ribavirin died. The geometric mean time to death for control animals was 6.1 days and for treated animals, 15.6 days. Viremia levels were significantly reduced (84-99.9% reduction, $p = 0.01$) in those animals which were treated with ribavirin.

One very striking difference between control and treated animals was observed by immunofluorescent staining of tissues from moribund animals. Viral antigen was not detected in the central nervous system in untreated animals. In contrast, the central nervous system of drug-treated animals was heavily infected with yellow fever virus as determined by immunofluorescence. Virus antigen was found in neuronal cell bodies and in processes projecting from the cell body as these processes transversed brain

These data suggest that animal given ribavirin died of a syndrome different from that of untreated animals. This type of altered pathogenesis, the late-stage entry into the CNS, has been seen in other virus-infected primates treated with ribavirin.

Alpha-beta interferon levels were low in both groups of animals with no differences observed between the two groups. One untreated monkey (87) had detectable levels (1:150) beginning on the 5th day after infection and lasting until the seventh day. Interestingly, this was a surviving monkey. However, among ribavirin-treated animals, only one of three surviving animals developed detectable levels of interferon (day 6-8).

c. Conclusion and hypothesis

Clearly, interferon development was late developing in ribavirin-treated animals, suggesting that interferon-stimulating drugs or other immunomodulating drugs might be of benefit in preventing death in ribavirin-treated animals. This hypothesis lead us into studies of immunomodulators.

B. YELLOW FEVER AND IMMUNOMODULATORS

1. Background and statement of the problem

In contrast to a conventional antiviral that inhibits a particular enzyme not universally present among all viruses, an immunomodulator can provide protection against a broad spectrum of viral infections by evoking the specific and nonspecific antiviral defenses of the host. Therefore, the identify of the etiologic agent is not relevant for the commencement of the treatment regimen. CL246738 (AVS 1968), an acridine hydrochloride (AH), evokes complete protection in rodents against viral infections representing 3 out of 4 arbovirus families including a flavivirus. In addition, Compound AVS 1018, a quinolinamine (QA) free base, administered in 2% lactic acid solution, evoked excellent protection against lethal viral infections representing both Bunyavirus and Flavivirus families. Therefore, these two drugs were tested in the yellow fever primate model for efficacy in preventing disease symptoms.

2. Results with CL246738 (AH, Lederle)

The prophylactic potential of AH was evaluated in squirrel monkeys that have natural resistance to the neurotropic strains of yellow fever virus infection; therefore, the circulating virus titer was the main indicator of the antiviral reactivity of this compound. Orally administered AH was given 24 h prior to yellow fever virus challenge. The compound evoked a marked antiviral activity by entirely reducing 3 logs of circulating virus titer. The compound was not effective when the treatment was initiated 24 h postinfection.

a. AH and Interferon

Serum from animals treated with AH was assayed for alpha/beta interferon. The details of the procedure were presented in Annual Reports (1989, 1990) and are summarized as follows. Test sera were diluted ten-fold in DMEM and adjusted to pH 2 by addition of HCl, held for 24 hours, and readjusted to pH 7.3. Half-log dilutions of each serum were incubated with Vero cells for 18 hours prior to infection of cells with VSV-Indiana using a dose which was 100 PFU's.

...did not produce uniform levels of interferon in monkeys until several doses of drug had been given, i.e., on days -1 and +1. Twenty-four hours after the first dose of drug, only one of four animals had detectable interferon. By day 2, all animals were positive. Interferon levels remained high through day 14, the end of the treatment schedule. These data suggest a reason for lack of drug efficacy when treatment began after virus inoculation. At least two days of drug treatment are required to induce detectable interferon.

b. Results of Combined Treatment with AH and Ribavirin

Addition of AVS #1 to the experimental protocol resulted in an initial depression in viremia, although later viremia levels were uniformly higher than those seen in animals treated with AH alone.

Drug treatment beginning after virus exposure produced no detectable differences from control animals exposed to virus alone. Viremia levels were similar in the two groups.

3. Results of Treatment with Quinolinamine (QA)

In cynomolgous monkeys, when QA was administered (orally) beginning one day before infection with the Asibi strain of yellow fever virus and given every other day for 14 days, 8 logs of serum virus titer were completely reduced. Although two of the three untreated infected monkeys died of yellow fever virus infection, all four treated monkeys survived the yellow fever virus challenge.

Table 5. Prophylactic Efficacy of Free Base (AVS-1018) in Yellow Fever Virus-Infected Cynomolgus Monkeys.

Group	Treatment	No. of Animals that Survived/Total
1	Virus ^a control	1/3
2	Virus ^a + quinolinamine ^b	4/4
3	Drug control	2/2

^a10³ PFU yellow fever virus injected i.m. on day 0.

^b20 mg/kg quinolinamine of day 1; 10 mg/kg on days 0,2,4,6,8,10,12,14 orally in 2% Klucel.

^cTreatment same as in group 2, without virus.

a. Quinolinamine (QA) and Interferon $\alpha\beta$

It may be significant that interferon in the serum of virus-infected, placebo-treated animals never exceeded 100 units at any time during the study. Those animals which died had low levels on day 1 and never again during the study except for one monkey which had interferon at the time of death (day 5). All virus-infected monkeys given drug in the study had detectable serum interferon on days 5, 7, 8, 10, and 12. It seems apparent from these results that the combination of virus-infection and drug treatment resulted in more consistent and prolonged interferon response than was observed in animals given drug only. Alternatively, the kinetics of the interferon response may have differed between those animals given both virus and drug and those given drug only.

b. Quinacrine and Yellow Fever Virus Viremia.

The effect of drug-treatment was most obvious on the suppression of viremia. As measured in Vero cells, the drug effect was dramatic. Only the virus-infected animals had a viremia. Only one virus-infected, drug-treated animal had a viremia, and it was transient, detected only on day 3 after infection. Viremia was not detected in the serum of other virus-infected, drug-treated animals. As expected, animals which received drug only did not have a viremia.

C. VACCINIA VIRUS IN PRIMATES

Two cynomolgous monkeys which survived the yellow fever virus study were held for three weeks prior to multi-site intradermal inoculation with wild-type vaccinia virus (5 sites, 0.1 ml, Koppe strain) described previously report. These animals were not producing detectable interferon and their blood chemistries and CBCs were normal at the time of virus inoculation. Lesions, which were too numerous to count accurately because of overlapping, developed on the chest at the site of subcutaneous inoculation seven days after inoculation resolved between 10 to 12 days after inoculation. Smears from vesicular scrapings done on day seven were positively stained by immunofluorescence using vaccinia virus antibody (and CCHF virus control antibody) prepared in mice. Multinucleate giant cells containing intranuclear inclusions were also identified in lesions on day 7. Infectious virus (between 10^3 and 10^4 TCID₅₀ per ml, 6 samples, 3 replicates each) was isolated in Vero cells from scrapings taken on days 7 and 8 only.

1. Effect of ribavirin on vaccinia lesions in primates

Six weeks after conclusion of the yellow fever virus experiment, four surviving monkeys were inoculated subcutaneously with vaccinia virus in the dosage described above. Two animals were treated orally with ribavirin, 100 mg/kg daily beginning one day before inoculation of virus. On day 7, lesions appeared on the chest of both the control and drug-treated animals. Smears from vesicular scrapings were antigen-positive by immunofluorescence on day 7. Multinucleated giant cells were not seen in the ribavirin-treated animals. Infectious virus, and the virus titer from the scrapping was lower (6 samples, 3 replicates each) in the ribavirin-treated animals (mean titer, $10^{1.7}$ /ml) as compared to the untreated controls (mean titer, $10^{3.3}$ /ml).

D. DISCUSSION AND CONCLUSIONS

Ribavirin was the antiviral studied in the yellow fever virus primate model. The therapeutic index of ribavirin *in vitro* for flaviviruses is lower than for bunyaviruses; no antiviral effect has been demonstrated in rodent flavivirus models. In other experiments, prophylactic administration of ribavirin to monkeys failed to abrogate dengue viremia³⁵ or to influence the course of yellow fever infection [Peters et al., Proc. Int. Symp. on Yellow Fever and Dengue, Rio de Janeiro 1988; Oswaldo Cruz Inst. 1990]. In our studies, ribavirin treatment markedly reduced viremia in monkeys although later invasion of the central nervous system was seen. This lead to a study in which we combined ribavirin treatment and immunomodulator therapy. Combined treatment did not reduce viremia significantly whereas treatment with either ribavirin or AH individually did reduce significantly viremia. This failure may result from the complexities of immunomodulator therapy (reviewed in [34]).

The two immunomodulators, AH and QA, studied in this model are not double-stranded RNAs; they apparently stimulate a variety of biological functions, induction of INF being most obvious. This is based upon the fact that administration of anti-INF serum abrogates their effect³⁴. A two-armed pathway appears to provide the antiviral mechanism of action. Via the upper arm, these immunomodulators stimulate INF release by macrophages, T lymphocytes, and fibroblasts. The INF in turn activates enzymes which inhibit viral proliferation. Via the lower arm, INF stimulates the specific and non-specific cellular or humoral immune reactivity. In our squirrel monkey studies, ribavirin not only reduced viremia, but it also delayed the onset of neutralizing antibody responses. Furthermore, only 1/5 animals treated with both ribavirin and AH (AVS 1968) had neutralizing antibody on day 7 whereas 4/5 placebo and 5/5 treated with AH alone had antibody on day 7. Thus, there may be antagonism between ribavirin and immunomodulator effects in primates. This is a question which should be further investigated.

The vaccine primate model deserves further exploration. Ribavirin treatment did not eliminate lesion formation. However, the virus titers in vesicular scrapings from the lesions were lower than in untreated controls and multinucleated giant cells were not seen in the scrapings. Whether giant cells are a normal aspect of vaccinia infection is unknown from the available literature; such cells are often seen in herpes virus infection. The virus isolated was vaccinia virus as determined by neutralization tests; we had no evidence of a concurrent herpes infection as determined by immunofluorescence tests. In conclusion, while ribavirin treatment did not eliminate or reduce lesion formation, it did reduce the virus titer in the vesicular scrapings.

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B. OTHER PUBLICATIONS WHICH PRESENT DATA COMPLETED IN THIS CONTRACT

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